Comparison of leaf terpenoids and tannins in *Juniperus osteosperma* from woodrat (*Neotoma stephensi*) browsed and not-browsed trees

Robert P. Adams
Biology Department, Baylor University, Box 97388, Waco, TX 76798, USA Robert_Adams@baylor.edu

Michele M. Skopec
Department of Zoology, Weber State University, Ogden, UT, 84405

and

James P. Muir
Texas A&M AgriLife Research, 1229 N. U.S. Hwy 281, Stephenville, TX 76401

ABSTRACT

Neotoma lepida (woodrat) browses on the leaves of *Juniperus osteosperma* near Dugway, UT. A comparison between woodrat (*N. lepida*) browsed and not-browsed *Juniperus osteosperma* trees revealed that the percentage of total volatile leaf oil yields was not significantly different between browsed trees (2.22%, 24 hr dist., DM-basis) and not-browsed trees (2.47%). On a percent total oil basis, α-pinene (4.5, 3.0%) was highly significantly higher in browsed trees, while α-campholenal (1.1, 1.3%) was significantly higher in not-browsed trees. On a mg/g DW basis, α-campholenal (0.23, 0.33%) and four compounds [p-cymene (0.34, 0.57), sabina ketone (0.20, 0.30), terpinen-4-ol (1.74, 2.67) and p-mentha-1,4-dien-7-ol (0.17, 0.25)] were significantly higher in not-browsed trees. There was a trend (P=0.075) for protein-precipitable phenolics (PPP) concentrations to be lower (3.64 mg/ g) in browsed than not-browsed (7.68 mg/ g). There was also a trend (P=0.081) for nitrogen content to be higher in browsed (0.76%) than not-browsed (0.67%). ADF (acid detergent fiber) was non-significant and averaged 27.33%. Published on-line www.phytologia.org *Phytologia 98(1): 17-25 (Jan. 5, 2016).*

KEY WORDS: *Juniperus monosperma, Neotoma stephensi,* woodrats, browsing, terpenes, protein-precipitable phenolics (PPP), nitrogen, ADF (acid detergent fiber) diet selection.

Populations of *Neotoma lepida* in the Great Basin utilize *J. osteosperma* for both food and shelter (Stones & Hayward 1968). Recent evidence suggests that one population in White Rocks Utah may actually specialize on *J. osteosperma.* with fecal pellet analysis showing >90% of plant fragments present to be *J. osteosperma* (unpublished observation, M. Skopec). Juniper foliage is visible in midden entrances (Fig. 1) and evidence of herbivory is present on many trees in the area (Fig. 2). However, the removal of foliage is non-random from adjacent trees (Fig. 2), suggesting that the woodrats are making foraging decisions, perhaps avoiding trees high in terpenes, similarly to another pine specialist, *Sciurus abert* (Abert's squirrel, Snyder 1992) or phenolics. *Neotoma stephensi,* a closely related specialist on *J. monosperma,* shows a similar foraging style on juniper and analysis of the terpene profiles of browsed and not-browsed junipers revealed that only one terpene, p-cymene, was found in higher concentration in not-browsed compared to browsed junipers, suggesting that *N. stephensi* is making foraging decisions based not on avoiding high levels of terpenes but perhaps seeking out higher nutrient content, or closer proximity to middens (Adams et al. 2014a). While much analysis of *N. stephensi's* physiological adaptations that allow it to metabolize the terpenes present in *J. monosperma* have been done (Boyle & Dearing, 2003; Dearing, McLister, & Sorensen, 2005; Haley, Lamb, Franklin, Constance, & Dearing, 2007; McLister, Sorensen, & Dearing, 2004; Skopec & Dearing, 2011; Skopec, Haley, & Dearing, 2007; Sorensen, Turnbull, & Dearing, 2004; Torregrossa, Azzara, & Dearing, 2011) very few studies have been conducted on mechanisms that *N. lepida* may utilize for terpene metabolism (Magnanou, Malenke &

Dearing, 2009; Skopec, Malenke, Halpert & Dearing, 2013; Wilderman et al., 2014). If analysis of browsed versus not-browsed *J. osteosperma* for differences in terpene and nutrient content reveal that *N. lepida* does not avoid terpenes like *N. stephensi,* more detailed analysis of *N. lepida* physiological mechanisms for metabolizing terpenes may be warranted.

Figure 1. Midden entrance. Note juniper leaves at the entrance to the midden/

Fig. 2. Not-browsed (left) and browsed (right) *J. osteosperma* trees near woodrat middens in Utah.

Considering the amount of research on the specialist woodrat (*N. lepida*), it is surprising that we could find no publication concerning the composition of *J. osteosperma* leaves from browsed trees vs. not-browsed trees. Although it should be noted that Adams (1994, 2012, 2013a, 2013b) and Adams and Kauffmann (2010) have published several studies of geographic variation in the leaf essential oils of *J. osteosperma* and on the effects of grinding leaves (Adams et al. 2014b). The purpose of this paper is to present new data on leaf volatile oils, protein-precipitable phenolics (PPP), nitrogen (N) and acid detergent fiber (ADF) from *J. osteosperma* leaves from *N. lepida* browsed and not-browsed trees.

MATERIALS AND METHODS

Plant material: *Juniperus osteosperma, Adams 14291-14300,* browsed trees, *Adams 14301-14310,* not-browsed trees, all common on and near granite, at White Rocks natural area, 7.4 mi n of Jct UT 199 and UT 196, thence 8 mi. w of UT 196. ~16 mi (25.7 km) nw of Dugway, UT, *40 19.367' N, 112 53.924' W,* 5254 ft (1567 m), 28 May 2014. Herbarium vouchers are deposited in the herbarium, Baylor University (BAYLU).

Essential oils analysis - A portion (200 g FW) of the fresh foliage was kept cool (20ºC) and in the dark, then, exhaustively steam-distilled for 24 h using a modified circulatory Clevenger-type apparatus (Adams 1991). Oil samples were concentrated (diethyl ether trap-removed) with nitrogen and stored at -20ºC until analyzed. Steam distilled leaves were oven dried to a constant weight (48 hr, 100ºC) for the determination of oil yield as [oil wt./(oil wt. + oven dried extracted foliage wt.)]. The extracted oils were analyzed on a HP5971 MSD mass spectrometer: 0.2 ul of a 10% solution (in diethyl ether) oil injected, split, 1:10, temperature programmed, linear, 60º - 246ºC at 3ºC/min. (62 mins.), carrier gas He, flow 34.96 cm/sec or 1.02 ml/min, injector 220ºC, detector 240ºC, scan time 1/sec, directly coupled to a HP 5890 gas chromatograph, using a J & W DB-5, 0.26 mm x 30 m, 0.25-micron coating thickness, fused silica capillary column (see Adams 2007, p. 4, for detailed operating conditions). Identifications were made by searches of our volatile oil library (Adams 2007) using HP Chemstation library search routines, coupled with retention time data of authentic reference compounds. Quantification was by flame ionization detector on an HP 5890 gas chromatograph operated under the same conditions as the GCMS (above) using the HP Chemstation software.

Protein-Precipitable Phenolics (PPP) - Condensed tannins were purified for subsequent use as a standard from dried *J. osteosperma* leaves modifying the method described by Wolfe et al. (2008) using Sephadex LH-20 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Protein-precipitable phenolics (PPP) were measured according to Hagerman and Butler's (1978) scaled down method as modified to determine protein precipitability of condensed tannins in two duplicate crude plant extracts (Naumann et al., 2013).

Nitrogen determination (N) - N (X 6.25 = crude protein) concentration. Samples were assayed for N concentration by combustion using an Elementar vario Macro C:N analyzer (Elementar Americas, Inc, Mt. Laurel, NJ, USA).

Acid Detergent Fiber (ADF) - ADF was determined by methods described originally by Van Soest et al., (1991) using an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY, USA).

Statistical analyses - Terpenoids (as percentage of total oil and as mg per g dry foliage weight), PPP, N, and ADF concentrations were compared between browsed and not-browsed samples by ANOVA and SNK (Student-Newman-Keuls) analyses as described by Steele and Torrie (1960). Differences were considered significant at $P \leq 0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

A detailed compositional analysis of *J. osteosperma* volatile leaf oils from browsed and not-browsed trees is shown in Table 1. ANOVA of the leaf volatile oils components (% total oil basis) for browsed and not-browsed trees revealed the percentage of total volatile leaf oil yields was not significantly different between browsed trees (2.22%, 24 hr dist., DM-basis) and not-browsed trees (2.47%). The lack of a significant difference in the yields of volatile oils was surprising. However, it is instructive to compare browsing (mostly goats) on two juniper species growing in the same population. For *J. ashei*, Adams et al. (2013a) found the browsers selected for lower leaf oil yield. But, in a companion study of browsed *J. pinchotii* (in the same population with *J. ashei* in the 2013a study), Adams et al. (2013b) found no significant difference in % oil yield between browsed and not-browsed trees. The closely related juniper specialist, *N. stephensi,* also seems to not make foraging decisions based on total amount of volatile oils (Adams et al. 2014a).

On a percent total oil basis, α-pinene (4.5, 3.0%) was highly significantly different and α-campholenal (1.1, 1.3%) significantly different between browsed and not-browsed trees. On a mg/g DW basis, α-campholenal (0.23, 0.33%) was highly significantly different and four compounds [p-cymene (0.34, 0.57), sabina ketone (0.20, 0.30), terpinen-4-ol (1.74, 2.67) and p-mentha-1,4-dien-7-ol (0.17, 0.25) were significantly different. Notice that four (of five) of these terpenoids are oxygenated (alcohols, an aldehyde and a ketone). Oxygenated compounds are generally more bio-reactive than hydrocarbons. The only terpene, α-pinene, that was found to be higher in browsed trees, is the major terpene in *N. stephensi*'s preferred plant *J. monosperma*, where it is found in levels 3-4 times that in *J. osteosperma* (Adams, Skopec, & Muir 2014). It is likely that *N. lepida* is able to effectively metabolize the lower concentrations of α-pinene found in *J. osteosperma.* Also a potentially interesting idea may be that *N. lepida* is actually seeking out α-pinene as a cue for trees that are lower in the oxygenated compounds, which may be more toxic. Based on these results it seems that *N. lepida* is making foraging decisions to avoid specific terpenes present in *J. osteosperma.* This pattern of not avoiding an entire class of PSC's, but only specific potentially bioactive members of a class of PSC's has been seen in other dietary specialists like the koala and pygmy rabbit (Moore & Foley, 2005; Ulappa et al., 2014).

There was a trend for protein-precipitable phenolics (PPP) concentrations to be lower (3.64 mg/ g) in browsed than not-browsed (7.68 mg/ g) trees (Table 2). If PPP (cf. tannins) interfere with digestion or decrease palatability, selecting trees with less PPP might be favored by woodrats (Bernays, Elizabeth, Cooper-Driver, & Bilgener, 1989; Haslam, 1989). There was also a trend for nitrogen concentration to be higher in browsed (0.76%) than not-browsed (0.67%), trees (Table 2). Selecting trees with higher

nitrogen might be expected but higher nitrogen can also be a result of younger material in regrowth points (Assefa et al., 2008; Reynolds et al., 1992). ADF varied little and was non-significant (Table 2).

Table 2. Protein-precipitable phenolics (PPP), Nitrogen and Acid Detergent Figer (ADF) for leaves of *J. osteosperma* (browsed by woodrats and not-browsed), Dugway, UT. ns = not significant at P= 0.05.

	browsed	not-browsed	F ratio	F significance
Protein-precipitable phenolics (PPP)	3.64 mg/g	7.68 mg/g	3.497	P = 0.075 ns
Nitrogen	0.76 %	0.67 %	3.337	P= 0.081 ns
Acid detergent fiber (ADF)	27.05 %	27.61 %	0.568	P= 0.533 ns

Principal coordinates (PCO)using 12 terpenes (mg/g) and oil yield (mg/g) data revealed an interesting pattern (Fig. 3). The trees appear to be in two groups, but not all browsed or not-browsed trees are in one group. Trees that were heavily browsed (Fig. 2, dashed line on right) are readily recognized. And even light browsing on a tree can be easily identified by the approximately 45° angle of the branchlet cut. It is likely, however, that trees may be lightly browsed on the top, and this browsing not visible from the ground. Thus, some trees are likely classed as not-browsed, when in fact they are being browsed (note four not-browsed trees within the dashed line ellipse with browsed trees, Fig. 3). In addition, it seems possible that a few trees may be sampled by woodrats and the cut branch discarded because it does not meet the woodrat's selection criteria (note one browsed tree within solid line ellipse with not-browsed trees, Fig. 3).

It is tempting to re-classify the trees based on oils and re-analyze the statistics, but that is not statistically valid. Greater attention to field identification of browsed and not-browsed trees may resolve this issue. Unfortunately, the trees sampled were not tagged, so we can not reexamine the trees in the field. Another difficulty in collecting was the lack of not-browsed trees in the area near the largest middens. Thus, it was necessary to move away from the midden(s) to find enough trees that were 'not-browsed'. If we inadvertently got out of the home range of the woodrats, some of the 'not-browsed' trees may not have been subject to browsing selection by woodrats. Male and female *N. lepida* were found to move only 252 and 136 ft on average from their middens a night in a similar habitat (Stones & Hayward, 1968).

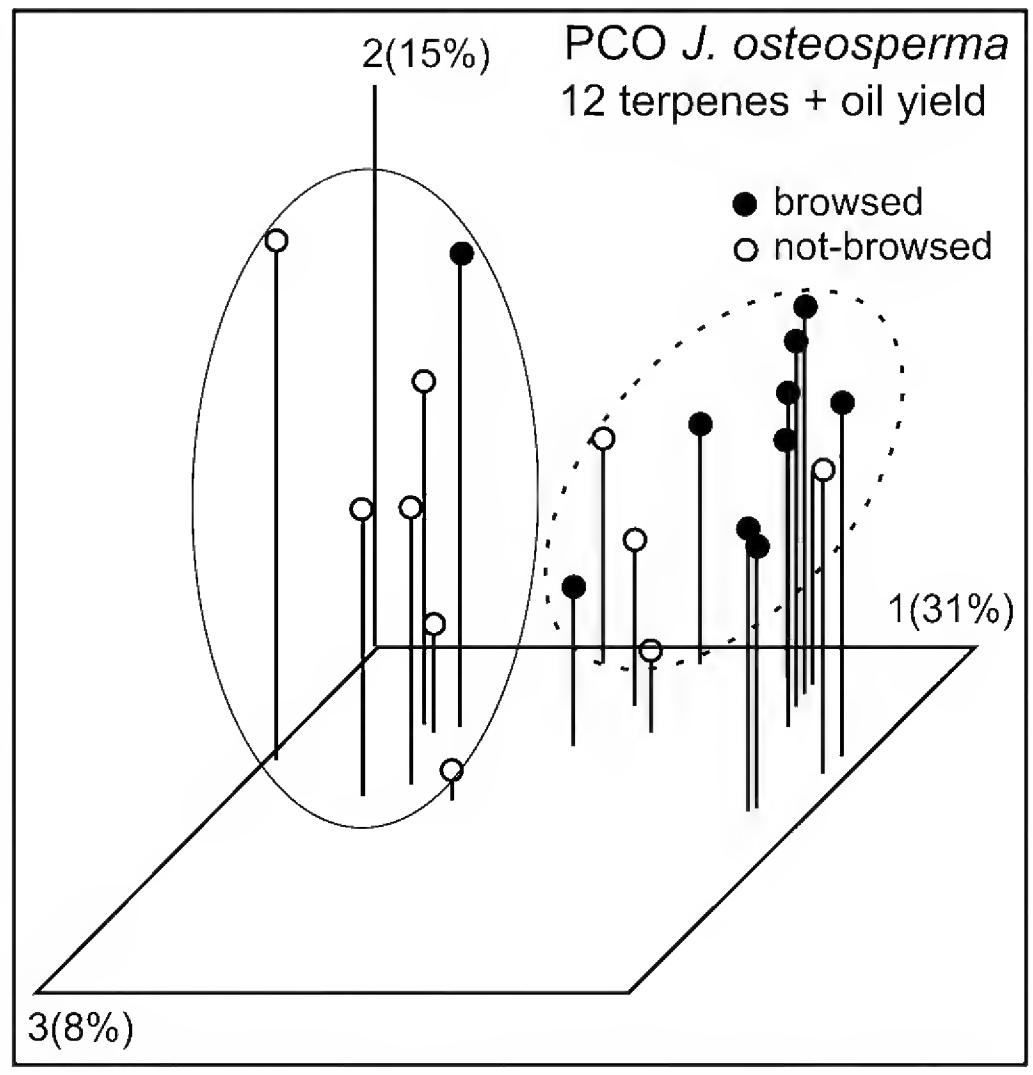

Figure 3. PCO of *J. osteosperma* trees browsed and not-browsed by woodrats. Ordination based on 12 terpenes (mg/g) and oil yield (mg/g) with character matches weighted by {[square root (F+1)]-1}. Where F is from ANOVA between browsed (10) and not-browsed (10) trees.

Compared to *N. stephensi*, that did not make foraging decisions based on terpene or tannin content, *N. lepida* seems to be choosing plants lower in oxygenated compounds and tannins and higher in α-pinene and protein (Adams et al., 2014a). While nutrient content of *J. monosperma* browsed by *N. stephensi* has not been measured based on results here and other studies with dietary specialists it is likely that *N. stephensi* do make foraging decisions based on nutrient density of the foliage (Moore & Foley, 2005; Schmalz, Wachocki, Wright, Zeveloff & Skopec, 2014; Ulappa et al., 2014).

In summary, analyses of browsed and not-browsed *Juniperus osteosperma* trees revealed that the percentage of total volatile leaf oil yield was lower, but not significantly different between browsed trees (2.22%, 24 hr dist., DM-basis) and not-browsed trees (2.47%). On a percent total oil basis, α-pinene (4.5, 3.0%) was significantly higher and α-campholenal (1.1, 1.3%) significantly lower in browsed versus not-browsed trees. On a mg/g DW basis, α-campholenal (0.23, 0.33%) and four compounds [p-cymene (0.34, 0.57), sabina ketone (0.20, 0.30), terpinen-4-ol (1.74, 2.67) and p-mentha-1,4-dien-7-ol (0.17, 0.25)] were significantly higher in not-browsed trees. There was also a trend for protein-precipitable phenolics (PPP) to be lower (3.64 mg/ g,7.68 mg/ g) and nitrogen concentration to be higher in browsed (0.76%) than not-browsed (0.67trees. ADF varied little and was non-significant. Taken together, it seems that *N. lepida* are making foraging decisions based on avoidance of PSM's and maximizing nitrogen intake.

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Table 1. Leaf essential oil compositions (% total oil basis and mg/g basis) for *J. osteosperma* (browsed and not-browsed), Dugway, UT. * = P 0.05, ** = P 0.001, ns = not significant at P= 0.05.

KI	Compound	browsed % total oil	not-browsed % total oil	F ratio, signif.	browsed mg/g	not-browsed mg/g	F ratio, signif.
	% oil & mg/g yield	2.22 %	2.47 %	1.48 ns	22.2	24.7	1.48 ns
846	(2E)-hexenal	0.3 %	0.3 %	nt	0.07	0.07	nt
921	tricyclene	0.5	0.6	nt	0.11	0.14	nt
924	α-thujene	0.3	0.3	nt	0.07	0.07	nt
932	**α-pinene**	**4.5**	**3.0**	**8.11** **	0.98	0.76	2.61 ns
946	camphene	0.6	0.6	nt	0.13	0.14	nt
953	thuja-2,4-diene	0.2	t	nt	0.04	t	nt
969	sabinene	5.4	5.3	0.19 ns	1.17	1.27	0.18 ns
974	β-pinene	0.1	t	nt	0.02	t	nt
988	myrcene	1.2	0.9	2.52 ns	0.26	0.23	0.55 ns
1002	α-phellandrene	0.2	0.2	nt	0.04	0.04	nt
1014	α-terpinene	1.0	1.2	0.57 ns	0.22	0.28	2.18 ns
1020	**p-cymene**	1.6	2.5	2.42 ns	**0.34**	**0.57**	**5.98** *
1024	limonene	2.5	2.0	3.34 ns	0.56	0.49	0.61 ns
1025	β-phellandrene	1.7	1.9	0.62 ns	0.38	0.48	2.06 ns
1044	(E)-β-ocimene	t	t	nt	t	t	nt
1054	γ-terpinene	1.6	1.9	0.68 ns	0.36	0.46	2.56 ns
1065	cis-sabinene hydrate	0.9	1.0	0.00 ns	0.21	0.24	0.46 ns
1067	cis-linalool oxide	t	t	nt	t	t	nt
1078	camphenilone	t	t	nt	t	t	nt
1086	terpinolene	0.9	0.8	0.05 ns	0.19	0.20	0.48 ns
1098	trans-sabinene hydrate	1.2	1.3	0.09 ns	0.27	0.31	0.74 ns
1102	isopentyl-isovalerate	t	t	nt	t	t	nt
1112	3-me-3-buten-me-butanoate	0.3	t	nt	0.07	t	nt
1118	cis-p-menth-2-en-1-ol	t	t	nt	t	t	nt
1122	**α-campholenal**	**1.1**	**1.3**	**6.21** *	**0.23**	**0.33**	**14.27** **
1141	camphor	21.9	21.7	0.01 ns	5.19	5.52	0.09 ns
1141	verbenol	11.0	11.1	0.00 ns	2.60	2.80	0.14 ns
1145	camphene hydrate	1.8	1.3	2.10 ns	0.38	0.33	0.94 ns
1154	**sabina ketone**	0.9	1.2	2.13 ns	**0.20**	**0.30**	**4.42** *
1160	pinocarvone	0.2	0.1	nt	0.04	t	nt
1165	borneol	4.5	5.3	0.86 ns	0.93	1.38	3.41 ns
1174	**terpinen-4-ol**	8.1	11.4	2.07 ns	**1.74**	**2.67**	**5.47** *
1179	p-cymen-8-ol	0.8	0.9	0.77 ns	0.18	0.22	1.83 ns
1186	α-terpineol	0.6	0.6	0.04 ns	0.12	0.14	1.93 ns
1195	myrtenol	0.2	0.2	nt	0.04	0.05	nt
1204	verbenone	1.6	1.2	1.67 ns	0.33	0.30	0.34 ns
1215	trans-carveol	1.6	1.3	1.05 ns	0.33	0.33	0.00 ns
1219	coahuilensol, me-ether	0.3	t	nt	0.07	t	nt
1223	citronellol	t	t	nt	t	t	nt
1226	cis-carveol	0.4	0.3	nt	0.09	0.07	nt
1238	cumin aldehyde	0.3	0.4	nt	0.07	0.09	nt
1239	carvone	0.8	0.8	0.92 ns	0.18	0.19	0.04 ns
1283	α-terpinen-7-al	t	t	nt	t	t	nt
1284	bornyl acetate	10.0	8.6	0.43 ns	2.19	2.15	0.01 ns
1298	carvacrol	0.6	0.5	1.25 ns	0.14	0.11	0.48 ns
1325	**p-mentha-1,4-dien-7-ol**	0.8	1.1	1.79 ns	**0.17**	**0.25**	**5.21** *
1468	pinchotene acetate	0.4	0.2	nt	0.08	0.05	nt
1513	γ-cadinene	t	t	nt	t	t	nt

KI	Compound	browsed % total oil	not-browsed % total oil	F ratio, signif.	browsed mg/g	not-browsed mg/g	F ratio, signif.
1522	δ-cadinene	t	t	nt	t	t	nt
1548	elemol	1.1	0.9	0.42 ns	0.23	0.23	0.01 ns
1574	germacrene-D-4-ol	t	t	nt	t	t	nt
1582	caryophyllene oxide	t	t	nt	t	t	nt
1627	1-epi-cubenol	t	t	nt	t	t	nt
1630	γ-eudesmol	t	t	nt	t	t	nt
1644	epi-α-muurolol	t	t	nt	t	t	nt
1649	β-eudesmol	t	t	nt	t	t	nt
1652	α-eudesmol	t	t	nt	t	t	nt
1652	α-cadinol	t	t	nt	t	t	nt
2312	abieta-7,13-diene-3-one	t	t	nt	t	t	nt

KI = linear Kovats Index on DB-5 column. Compositional values less than 0.1% are denoted as traces (t). Unidentified cpds. less than 0.5% are not reported.